



Saskatchewan
Agriculture
and Food

ADF

**AGRICULTURE
DEVELOPMENT
FUND**

FINAL REPORT

20010116

**GROWTH, SURVIVAL AND CONTROL OF FOODBORNE
PATHOGENIC BACTERIA ON PROCESSED MEAT AS
INFLUENCED BY INTRINSIC PARAMETERS**

Funded by: The Agriculture Development Fund

March, 2005

Prepared by: University of Saskatchewan

2007-04649/

**Growth, Survival and Control of Foodborne
Pathogenic Bacteria on Processed Meats**

ADF Project #20010116

Final Report

March 2005

George G. Khachatourians

TABLE OF CONTENTS

EXECUTIVE SUMMARY	3
TECHNICAL REPORT	3
1. BACKGROUND	5
2. PROGRESS REPORT	
2.1. Chelation as a Means of Meat Preservation	6
2.2. Antioxidants as a Means of Meat Preservation	
2.2.1 Introduction	6
2.2.2 Characteristics of Antioxidants	7
2.3 Antioxidants of Natural and Synthetic Origins	8
2.4 Synthetic Antioxidants	8
2.5 Natural Antioxidants	9
2.5.1 Ascorbic acid	10
2.5.2 Origanox and other natural compounds	10
2.6 Antimicrobial Activity of Antioxidants	11
2.6.1 <i>Listeria monocytogenes</i>	12
2.6.2 <i>Campylobacter jejuni</i>	12
3. OBJECTIVES	13
4. MATERIALS AND METHODS	13
4.1 Bacterial Strains	13
4.2 Growth and Survival Tests	13
4.3 Meat Tests	14
5. RESULTS and CONCLUSIONS	15
5.1 Determination of Chelator Effect	15
5.2 Determination of MIC in Growth Media	15
5.3 Determination of MIC on Processed Meats	16
6. FUTURE PLANS	17
7. REFERENCES	17
8. PERSONNEL CONTRIBUTING TO THIS PROJECT	20
9. STATEMENT OF EXPENDITURES	20

EXECUTIVE SUMMARY

The overall objectives of this research were to determine the roles played by some of the intrinsic physical and chemical parameters of processed meat, namely iron availability, fat content, osmolyte content and antioxidant levels on the growth and survival of pathogenic bacteria on meat products. The data obtained will be used to optimize these parameters in existing processed meat products so as to maximize product safety.

The project used two standard processed meat products, bologna and summer sausage, as a model meat system. In these meats, due to their intrinsic properties, among others, water activity and pH, allow for the survival of the pathogens without growth, which potentially under favored conditions will grow and proliferate. Two well-known bacterial pathogens commonly associated with processed meats and which are of concern in ready-to-eat meats, namely *Listeria monocytogenes* and *Campylobacter jejuni* were used as pathogens. Dr. Khachatourians' supervision of this project commenced in July of 2003 with Mr. Michal Dworaczek as project Technical Research Assistant. Mr. Dworaczek made a career change and left the project in September 30, 2003. This left Dr. Khachatourians' supervision with two BSA students, Misses Lindsay Mooney and Jolanta Pisz to work on the project between fall and summer of 2004.

During the previous reporting periods (October 2001 to March 2002 and April 2002 to January 2003), the following evaluations were performed and have been reported:

- (1) Growth kinetics of two *Listeria monocytogenes* strains *in vitro* against different concentrations of ferric ions and different growth temperatures;
- (2) The interaction between temperature, ferric ammonium citrate and glycine betaine on growth of *Listeria monocytogenes*;
- (3) The planktonic or attached growth of *L. monocytogenes* as influenced by temperature during pre-incubation on the bologna and not on the summer sausage;
- (4) Tests on growth of *C. jejuni* in the presence of ferric nitrate as the source of ferric iron, grown at 30, 37 and 42 °C with or without the addition of the osmolyte, glycine betaine in bologna and summer sausage;

For the last reporting period (January 2003-March 2005), the remaining evaluations were performed and will be reported here:

- (5) Tests on growth of *L. monocytogenes* or *C. jejuni* in bologna and summer sausage at refrigeration temperature of 4 °C in the presence of the chelator, EDTA, which removes divalent cations; and
- (6) Tests on growth of *L. monocytogenes* or *C. jejuni* in bologna and summer sausage containing two natural antioxidants (Ascorbic acid/ vitamin C and Origanox) and two synthetic food grade antioxidants (BHA and TBHQ).

The overall results permit the following conclusions. The effect of the chelator, EDTA, at 55.5, 166.6 and 500 ppm on the subsequent survival of *C. jejuni* during storage on chilled processed meats vacuum packaged and incubated at 4°C for 3 weeks was determined. Results indicated that under all three concentrations of EDTA the bacterial growth on the bologna showed less immediate reduction than those without the additive. Therefore, EDTA is of no practical value in final product formulation of these meats. These results also indicate that *L. monocytogenes* 4 is sensitive to processed meats treated with synthetic antioxidants, BHA, and to higher concentrations of TBHQ, but not to the natural ones, ascorbic acid or Origanox, even at extremely high concentrations. On the other hand *C. jejuni* 2 is very sensitive to treatment with TBHQ, and high concentrations of BHA, and sensitive to extremely high concentrations of ascorbic acid, but not Origanox.

TECHNICAL REPORT

BACKGROUND

There were two overall objectives, described as milestones 4 and 5 remaining for this research. These objectives were to determine the roles played by antioxidants and a chelating compound on the growth and survival of human foodborne pathogenic bacteria on our model meat products, and from the data obtained from this investigation to make recommendations to processed meat industry so as to maximize product safety.

Strains of two bacteria, *Listeria monocytogenes* and *Campylobacter jejuni*, both of which are of concern in ready-to-eat meats, but which display very different physiological requirements were employed as pathogen models. *Listeria monocytogenes* is a Gram-positive, widely distributed, air-tolerant pathogen with a low rate of infection but a high mortality rate in infected individuals. On the other hand *C. jejuni* is a Gram-negative, fastidious and oxygen-sensitive pathogen that causes a high number of cases of infection, but which is seldom fatal. These two bacteria are major human pathogens, which can be found on meats. Two standard processed meat products, bologna and summer sausage were used as model meats. Among the differences in these meats are their intrinsic properties among others, water activity and pH, which allow for the growth of pathogens or the survival of the pathogens, without growth, respectively, and were used throughout the study.

PROGRESS REPORT

1. Chelation as a Means of Meat Preservation

There is not much known about the effects of chelating substances on the presence of free or attached forms of food borne pathogenic bacteria to processed meats. Walls et al. (1993) reported that *Salmonella typhimurium* cells association with sausage casings were inhibited when cells were incubated with glucose, galactose, hyaluronin, chondroitin sulfate, Tween 20, EDTA, gelatin, glycine and bovine albumin. There was a small decrease in attachment with increasing sodium chloride levels from 0-100 mg/ml.

2. Antioxidants as a Means of Meat Preservation

2.1 Introduction

The removal of oxygen and the addition of carbon dioxide have long been used to preserve meat and meat products. Antioxidants are compounds, which are naturally found in many foods. They can be added to processed foods as an extra assurance of product stability. They are especially useful preservatives in a situation where a decrease in oxygen concentration, low storage temperature, or higher degree of saturation are undesirable for the given food product (Pokorny, 1999). The anti-spoilage properties of antioxidants have always aided food preservation through minimizing the growth of spoilage organisms and pathogens. Only recently has the use of antioxidant in foods been researched in an effort to make broader and greater effort to make foods safer.

With the increase of cardiovascular diseases, more and more people are shifting towards unsaturated fatty acids in their diets. However, food with higher concentrations of unsaturated fatty acids is prone to oxidative breakdown. The more double bonds present, the more sensitive foods are to oxidative breakdown. A higher temperature is also a controlling factor in oxidative breakdown; a higher temperature can lead to the oxidation of even saturated fatty acids.

In the presence of oxygen, normal cellular metabolism undergoes autoxidation, creating free radicals. Some of these radicals are chemically reactive. They decrease the stability of the food ingredients, and affect the blood vessel walls, leading to rapid ageing, arteriosclerosis, and

cancer (Pokorny, 1999). During autoxidation ambient oxygen that is found in the air is converted into a reactive singlet form that is capable of inserting itself into the double bonds of unsaturated fatty acids, releasing a hydrogen atom, and creating a peroxy radical. These peroxy radicals can take up a hydrogen atom from another double bonded site, resulting in lipid hydroperoxides. Hydroperoxides cause great concern, as they are associated with mutations and toxicities (McCall and Frey, 1999). Most living organisms produce natural defense against the threat of autoxidation, but many times an additional material could augment defense, which is better. In the human body, lipophilic and hydrophilic antioxidants in combination with enzymes, like superoxide dismutases and catalases, protect the body from accelerated oxidative damage (McCall and Frey, 1999). Obviously meats containing fats are especially vulnerable to oxidative damage. Therefore it is not unusual to augment such meats with antioxidants.

2.2.2 Characteristics of Antioxidants

Once the autoxidation process begins, the rate of oxidation dramatically increases. Antioxidants prolong the time lag involved when oxidation is progressing. As a result oxidation progresses very slowly. The peroxy radicals that are formed by oxidized lipids react with the antioxidant forming a stable antioxidant radical and a hydroperoxide. The antioxidant free radicals do not participate in an oxidation chain, unless they are present in excess. Other lipidic peroxy radicals or alkoxy radicals can inactivate such molecules. Dimers or trimers formed by these types of radicals also exhibit antioxidative properties. In exceptional situations when antioxidants are present in excess, or when there is a high degree of unsaturation, peroxidation occurs (Pokorny, 1999).

Antioxidants used in the food industry, as antimicrobials and preservatives, are selected based on a number of different characteristics. The antioxidant used in food must have a non-detectable flavour, or colour, so as not to alter the quality of the food. It must be capable of dissolving in the food. Some antioxidants, like butylated hydroxyanisole (BHA), cannot dissolve in water, thus limiting the range of food that they can be mixed with. Both the composition of the water and the lipid fraction of the food must be considered (Pokorny, 1999). The stability of the antioxidant is critical, especially for foods that undergo long periods of storage or heating. The

overall structure of the antioxidant, as well as the structure of the food, will alter the activity of antioxidation.

Table 1. Some common antioxidants that are found in most foods.

Food Antioxidants*		
Ascorbic acid	BHA	Propyl gallate
Calcium ascorbate	BHT	Octyl gallate
Ascorbyl palmitate	TBHQ	Dodecyl gallate
Ascorbyl stearate	Sulphur dioxide	Gum guaiac
Sodium ascorbate	Tocopherols	Various sulphates
Sodium erythorbate	Erythorbic acid	

* Adapted from Schuler (1990)

2.2.3 Antioxidants of Natural and Synthetic Origins

Antioxidants in use for human applications are of two origins, natural and synthetic. While in functional sense both groups act to serve the same end purpose, they have unique variance in many other ways, e.g., levels of tolerance, toxicity and carcinogenicity, and applicability in terms of safety.

2.2.4 Synthetic Antioxidants

Synthetic antioxidants are usually more affordable, more reliable, and have stronger antimicrobial effects than natural antioxidants (Yen et al., 2001 and Pokorny, 1999). However these compounds are often criticized on the possible toxicity and carcinogenicity that they may produce. Phenolic derivatives have proven to be much safer than heterocyclic antioxidants (Pokorny, 1999). These antioxidants differ by the *p*-substituted alkyl group, which varies their toxicity and lipid solubility. Information on the toxicity of synthetic antioxidants is still limited; however the synthetic antioxidants seem to have a greater effect than natural antioxidants (Nenadis et al., 2003).

In this research we used two antioxidants. The synthetic antioxidant butylated hydroxytoluene (BHT) has been indirectly used in food and animal feed products, and directly used in cosmetic and pharmaceutical products (Williams et al., 1999). Butylated hydroxytoluene is very similar to BHA in terms of promotion or prevention of cancers in animals. These

compounds when used together in addition demonstrate antimicrobial synergism (Pokorny, 1999). Both of these synthetic compounds are soluble in oil emulsions but are insoluble in water.

Butylated hydroxytoluene has received a great deal of criticism, as it has been shown to act as both a carcinogen and an anticarcinogen (Iverson 1999, and Chung et al., 1999). The metabolism of BHA in the liver creates the metabolites tertiary butylhydroquinone (TBHQ) and tertiary butylquinone (TBQ) (Williams et al., 1999), both of which have carcinogenic properties. Butylated hydroxyanisole acts as a cancer promoter in mice, which have had a previous exposure to a known carcinogen, but if treated with BHA prior to exposure to a known carcinogen, BHA acts as an anticarcinogen (National Cancer Institute, 2004). It is known that cancer prevention by BHA functions by free radical trapping, and the activation of the metabolic enzymes involved in detoxification and conjugation processes (Williams et al., 1999, and Iverson, 1999). The dosage of the antioxidant seems to play a key role in the cancer promotion and cancer prevention activity. Concentrations of 250mg/kg/day have demonstrated dangerous carcinogenic properties in the laboratory, but this is much higher than normal consumption (Williams et al., 1999). The key finding in this regard is the approximate intake of BHA less than 0.1mg/kg/day by humans does not pose a risk of cancer promotion, but allows for it to act as an anticarcinogen. Thus, BHA is accepted as a GRAS (generally regarded as safe) compound and can be safely administered to food preparations.

It has been reported that BHA inhibits the growth of many bacteria, including members of the genera *Salmonella*, *Staphylococcus*, *Clostridium*, *Pseudomonas*, *Escherichia*, and *Vibrio* (Shelef and Liang, 1982). Those foods with higher fat contents should demonstrate a lower antimicrobial effect with treatment with BHA, because this antioxidant is fat-soluble. Higher levels of protein are also believed to reduce the antimicrobial action. Tests performed on rice and chicken showed that the chicken provided more resistance to the antimicrobial properties of BHA (Shelef and Liang, 1982). Foods with high fat and high protein contents, like many types of meat, would require a high concentration of BHA for an adequate microbial control.

2.2.5 Natural Antioxidants

A growing number of consumers are demanding naturally based food products. Natural antioxidants are more desirable for use in the food industry, as they are more aesthetically pleasing to consumers and they usually have a higher maximum allowable concentration than

synthetic antioxidants. The majority of natural antioxidants that are used in food preservation are also in the phenol form, but unlike synthetic antioxidants, they differ in the *ortho*- position (Pokorny, 1999). Natural antioxidants are often times more costly than the synthetic ones because of the complex extraction processes needed to obtain them. To make them more affordable an increasing number of natural antioxidants can be chemically synthesized (Pokorny, 1999). Natural antioxidants can be found in fruits and vegetables, and can be produced by fungi. *Aspergillus candidus*, *Penicillium roquefortii* and *Mortierella* species produce antioxidant products (Yen et al., 2001). Fruits, vegetables, and oilseeds containing natural antioxidants often lose them during processing. Weak antioxidants, such as tocopherols are present in oilseeds, which are often removed during the refining process, and must be added again to aid in preservation (Pokorny, 1999).

2.2.5.1 Ascorbic acid

There are many benefits to this water-soluble antioxidant. Ascorbic acid (vitamin C) has been correlated with reducing the risk of heart disease development, boosting the immune system, inactivating carcinogenic N-nitroso compounds that are found in the stomach and delaying cataract formation (Bermond, 1990). Ascorbic acid has also been associated with many side effects when consumed at high concentrations. Some of the adverse effects of an over-dosage of vitamin C, include enhanced rate of plasma clearance (conditioned scurvy), formation of kidney stones, vitamin B12 destruction, diarrhoea, erosion of dental enamel, copper loss, excessive iron uptake, and even peroxidative effects (Hathcock, 1995). The recommended daily allowance of ascorbic acid for adults is 30 to 100mg, differing from country to country (Bermond, 1990). We used two naturally occurring antioxidants, ascorbic acid and Origanox.

2.2.5.2 Origanox and other natural compounds

Origanox is a water-soluble, GRAS status antioxidant that contains multiple ingredients of herbal extracts, like rosmarinic acid (Barrington Chemicals, 2002). According to Barrington Chemicals, Origanox's antioxidative effect is three times greater than those of BHA, TBHQ, vitamin E and vitamin C. This heat stable compound is rather new, and little scientific data has yet been released on its antimicrobial nature. However, it has been shown that Origanox has significant inhibitory effects to the pathogens *Escherichia coli* O157: H7 and *Salmonella*

choleraesuis. The addition of only 0.1% (w/v) Origanox to BHI medium resulted in a minimum of a 2-log decrease in colony forming units, per millilitre (Dharmavaram et al., 2003).

There are mixed reviews over the benefits or adverse effects caused by tocopherols (vitamin E) in relation to heart disease. Moderate intake of tocopherols provides many benefits, but higher concentrations can also lead to problems. This vitamin is considered to have a low toxicity at high concentrations, however, connections between high intake of vitamin E and diseases like hyperlipidemia have been uncovered (Hathcock, 1995).

Flavonoids are another set of antioxidants that are found in honey and in both green and black tea. They promote the antioxidative action of glutathione and prevent the oxidative consumption of vitamins C and E. They also have anticarcinogenic implications, which have been demonstrated by studies performed on mice (Lin et al., 2000).

Carotenoids are natural antioxidants that are present in fruits and vegetables. They are responsible for giving them their bright colours. Animals cannot synthesize carotenoids on their own; carotenoid levels are dependent on the diet of the animal (Waldenstedt et al., 2003). Carotenoids are divided into two groups based on their chemical composition. Beta-carotene, which is a form of vitamin A, belongs to the group containing only carbon and hydrogen atoms. This carotene is linked to a decrease of cancer risk, but only with a moderate ingestion. There is one piece of evidence that beta-carotene supplementation to middle-aged male tobacco smokers, actually increased risk of cancer, but little else has been uncovered (Pryor et al., 2000 and Hathcock, 1995). The other group of carotenoids includes those compounds that contain carbon, hydrogen, and oxygen. These include compounds such as astaxanthin, lutein and zeaxanthine. Astaxanthin is responsible for egg yolk and salmon pigmentation and lutein and zeaxanthine are found in green leafy vegetables (Waldenstedt et al., 2003).

It is also known that combinations of natural antioxidants can have a higher potency and demonstrate a higher antimicrobial activity, than preparations of one antioxidant.

2.2.6 Antimicrobial Activity of Antioxidants

Listeria monocytogenes and *C. jejuni* are two pathogens that are of greatest concern in meat products that were proposed for this study. Their salient features are discussed below.

2.2.6.1 *Listeria monocytogenes*

Animal ingestion of *L. monocytogenes* results in serious disease, and can lead to infertility in young women. Meat is considered free of *Listeria* if treated with a high temperature, 70°C, for as little as two minutes (Beumer et al., 1996). However, if the meat is contaminated after the heat treatment, the cooked meat still provides a suitable environment for growth of this pathogen. The growth of *Listeria* on cooked meat differs only in the duration of lag phase, with variation dependent on the temperatures of storage (Beumer et al., 1996). Studies have indicated that the pathogen prefers a pH above 6, and grows very poorly at lower pH values (Glass and Doyle, 1989).

2.2.6.2 *Campylobacter jejuni*

Upon ingestion, *C. jejuni* causes major gastrointestinal upset even when only a small numbers of organisms are ingested. The growth of this pathogen is a threat in meat products, as it prefers the low oxygen environment that is often facilitated in meat packages. Though extremely sensitive to the high oxygen concentrations present in air, *Campylobacter* species have special enzymes to counter this situation (Park, 2002). *Campylobacter jejuni* contains an enzyme that converts free oxygen radicals into H_2O_2 and O_2 , which can occur during freeze thawing of meats. It also contains an enzyme that becomes activated in conditions where oxygen levels are below a metabolic threshold. This enzyme converts H_2O_2 into water and O_2 (Stead and Park, 2000). Both of these metabolic processes require iron. The addition of iron compounds, such as $FeSO_4$, can be used to scavenge the free oxygen radicals, and thus increase the oxygen tolerance of *Campylobacter* (Hänninen et al., 1984).

Campylobacter species are sensitive to differences in the pH of the meat and temperature of storage. It seems to grow much easier in meats above pH 6.4, and at temperatures of 37 C, with optimal growth at 42 C (Park, 2002). *Campylobacter* cell proliferation and hence numbers should not increase in the low temperatures during refrigerated storage. These pathogens are extremely sensitive to desiccation and sodium chloride concentrations above 2% (w/V) (Park 2002). Under physiological stress the cells' morphology changes from bacillary to a coccoid, and non-cultivable form (Talibart et al., 2000), which makes cultivation in a laboratory setting difficult. In spite of this, as small an infectious dose as 500 organisms, is needed in most meat products, to bring about a major health threat for the food industry (Park, 2002). Little is known

about the response of *Campylobacter* proliferation in antioxidant treated meats. Previous studies using carotenoids in broiler chicken feed have shown that while this method decreased *Clostridium perfringens* caecal colonization, *C. jejuni* colonization was unaffected (Waldenstedt et al., 2003).

3. OBJECTIVES

The objectives were to investigate the survival of *L. monocytogenes* and *C. jejuni* on bologna and summer sausages made up with different amounts of EDTA and *in vitro* survival of these bacteria in the presence of antioxidants on meats.

4. MATERIALS AND METHODS

4.1 Bacterial strains

One bacterial strain was used in this study. The *C. jejuni* was obtained from the Saskatchewan Institute of Applied Science and Technology (Saskatoon, Saskatchewan, Canada) and isolated from a case of human clinical campylobacteriosis. Two strains of *L. monocytogenes*, LRC4 and ATCC 19113 used were from our laboratory stocks.

4.2 Growth and survival tests

Commercial MOX (modified Oxford agar) or BFC (Blood Free Campylobacter selective agar) plates were purchased from VWR Canada. The synthetic medium was prepared according to Tenover et al. (1985).

Campylobacter jejuni was revived from -80°C to a CCDA plate and incubated at 37°C for 48 h in 5% oxygen and 10% CO₂ atmosphere. Then it was restreaked on another CCDA plate and incubation process was repeated. Following this it was inoculated into 100 ml of BHI (Difco) with 2% of Yeast Extract (Difco) and incubated at 37°C for 48 h in 5% oxygen and 10% CO₂ atmosphere. From there, 1 ml of the culture was transferred into 100 ml of defined medium and left to incubate at 37°C for 48 h in the same atmosphere as above. After 48 h the cells were harvested by centrifuging at 10000 rpm for 10 minutes. Cells were then resuspended in PBS and

this process was repeated two times. The culture was then finally adjusted to optical density of 0.4 at 600 (nm) on Novaspec II Visible Spectrophotometer.

The two strains of *L. monocytogenes*, LRC4 and ATCC 19113 were inoculated into modified Pine's medium when required containing 0.088-g/l ferric ammonium citrate. The growth was determined by readings of optical density at 600 nm at appropriate time intervals. At 4°C, but not other temperatures, increasing ferric ammonium citrate resulted in improved growth in the absence, but not the presence, of glycine betaine.

To perform bacterial survival tests on meats a 10g slice of either bologna or summer sausage was aseptically placed onto a sterile Petri dish. Each slice was spread with a pure culture of either bacterium, leaving one slice of bologna and one slice of summer sausage aside to use as a control. These were then incubated for 48 hours at 37 °C (those meats spread with *C. jejuni* 2 were incubated in a sealed gas pack jar). After incubation, the surfaces of the meats were wiped and spread onto either MOX (modified Oxford agar) or BFC (Blood Free Campylobacter selective agar) plates, depending on the inoculated organism. The control meats were spread on both MOX and BFC. These agar plates were then incubated at same conditions.

4.3 Meat tests

Custom sausages, bologna and summer sausage were prepared at the University of Saskatchewan Meat Pilot Plant, with 0, 55.5, 166.6 and 500 ppm of disodium EDTA from BDH Company. They were stored at -1°C, vacuum-packed until the time of experiment, when they were cut into 10-g slices using a commercial slicing machine. Experiments were done in triplicate; thus there were 3 slices per sampling of 0, 3 for 55.5, 166.6 and 500 ppm of EDTA. This gives in total of 12 slices.

Slices were aseptically stripped from casing and inoculated with 100 µl of *C. jejuni*. The cultures on the surface of the sausages were spread with a glass spreader and left to absorb for 5 minutes. The slices were then packed into nylon-EVA copolymer bags with stated oxygen permeability of 52 ml m⁻² atm⁻¹ 24 h⁻¹ at 23°C using Vacuboy (Komet, Plochingen, Germany) packager. The product was stored at 4°C and analyzed on weekly or daily basis, depending on the sausage type, by stomaching, dilutions and plating on Campylobacter blood-free selective agar base plates.

The pH of the summer sausage and the bologna was tested after stomaching. First the pH of plain peptone water was measured, after a two-point adjustment of the pH meter. Then the pH values of stomached (120 seconds) peptone and summer sausage, and peptone and bologna solutions were measured.

A flow chart on process and data collected are attached as Figure 1 for better understanding.

5. RESULTS and CONCLUSIONS

5.1 Determination of Chelator Effect

The effect of the chelator, EDTA, at 55.5, 166.6 and 500 ppm on the subsequent survival of *C. jejuni* during storage on chilled processed meats, vacuum packaged and incubated at 4°C for 3 weeks was determined. Results indicated that under all three concentrations of EDTA the bacterial growth on the bologna showed less immediate reduction than those without the additive. While there was a five-log reduction by chilling at 4°C in the first week for the bologna samples without the additive, those with 55.5, 166.6 and 500 ppm EDTA showed 4.6, 4.2 and 2 log reductions in bacterial survival respectively. On further incubation for an extended 2 or 3 weeks, the survival values were reduce by 6 to 7 logs, reaching 90 to 99 or 100% killing of the pathogen. These results indicate that addition of EDTA while chelating divalent cations which are important for the pathogen, provide at least a concentration dependent survival benefit. The situation was different for summer sausage. In this case, all three concentrations of the additive further reduced the survivability by an additional log compared to those samples without it. This effect was noticeable at first week of sampling. Overall, while the chelator, EDTA can be effective in further reduction of *C. jejuni* on summer sausage; it is of no practical value in final product formulation for bologna.

5.2 Determination of Minimum Inhibitory Concentrations in Growth Media

Broth cultures of *L. monocytogenes* and *C. jejuni* were streaked out onto Petri plates containing MOX or BFA media. A series of standard size filter papers were soaked in varying concentrations of the antioxidant Origanox, Ascorbic acid, BHA, and TBHQ concentrations and

placed on top of the inoculated plates. The plates were then incubated for 48 hours. The Minimum Inhibitory Concentration values for antioxidants were measured. Cells of the cultures of *L. monocytogenes* and *C. jejuni* showed inhibition of growth with a concentration of 6.25 and 50 ppm BHA and 25 and 6.25 ppm of TBHQ respectively. The natural antioxidants, ascorbic acid or Origanox had a minimum inhibitory effect on either bacteria at 5,000 ppm.

These results indicate that *L. monocytogenes* is sensitive to treatment with BHA, and to higher concentrations of TBHQ, but not to Ascorbic acid or Origanox, even at extremely high concentrations. On the other hand *C. jejuni* is very sensitive to treatment with TBHQ, and high concentrations of BHA; it also demonstrates sensitivity to extremely high concentrations of ascorbic acid, but not to Origanox.

5.3 Determination of Minimum Inhibitory Concentrations on Processed Meats

In the presence of antioxidants, the growth curve of a strain of *L. monocytogenes* was measured. The 10g slice of either bologna or summer sausage contained ascorbic acid at a concentration of 1,000 ppm, or Origanox at 2,000 ppm, or TBHQ at 200 ppm, or BHA at 200 ppm. In the third trial concentrations of 2,000 ppm and 5,000 ppm for ascorbic acid, 4,000 ppm and 10,000 ppm for Origanox, and 400 ppm and 1,000 ppm for BHA and TBHQ, were also used on the bologna slices.

A pure stock culture of *L. monocytogenes* stored at -80 C was revived on a Tryptic Soy agar plate and incubated at 37 C for 24 hours. A pure colony was then used to inoculate 100 ml of Tryptic Soy broth and this was left to incubate for another 24 hours at 37 C. One millilitre of the culture was then transferred into each of 10 separate micro centrifuge tubes and spun at 10,000rpm for 10 minutes. The cells were then re-suspended into PBS and centrifuged again at 10,000rpm for 10 minutes. Once an OD₆₀₀ of 0.4 was obtained the culture was inoculated onto the deli slices at 0.1ml/10g slice. The drop of culture was then spread onto the slice in the same area as the antioxidant solution, which was also dropped onto the surface of the meat at 0.1ml. The samples that were designated week 0 were immediately stomached for 2 minutes with 90ml of 1.0g/l peptone. These samples were then diluted into the appropriate dilutions. Dilutions used were based on those established in the previous trials. The drop plate technique was used, 4 drops of 0.025ml each. The plates were then incubated aerobically at 37 C for 24 hours, and the colonies formed were counted. The data were calculated by taking the average number of

colonies and multiplying by the dilution, and then dividing it by 10, to account for the mass of the meat. The results were then put in $\log_{10}$ form. See attachments for the data and graphs. The data consists of three replicate trials for statistical considerations.

On the antioxidant treated bologna there was little difference between the antioxidants in the initial week, but the control did not have as many colony forming units as the antioxidant treated meats. After the first week the number of colony forming units decreased, and stabilized between 3.0 and 5.5 average log CFU/g. The summer sausage was unable to support the growth of *Listeria* beyond 3 weeks. The antioxidant treated summer sausage did have lower numbers than the control, even at the highest point, week 1. The difference between the two types of meat accounts for the behaviour of the *Listeria* strain. The summer sausage tends to be saltier than the bologna, and has a much lower pH. This in combination with the electron snatching activity of the antioxidants may account for the low amount of colony forming units and short survival time.

6. FUTURE PLANS

The results obtained from the last phase, that is the effects of EDTA and antioxidants on *L. monocytogenes* and *C. jejuni* will be processed for a public presentation and possible publication in a meat science journal.

7. REFERENCES

- Barrington Nutraceuticals. (2002) Origanox™ Powerful Natural Antioxidant. *Analytical comparison between Origanox WS, vitamin C, vitamin E, BHA and TBHQ emulsions*. [Electronic version] Retrieved June 3rd, 2004, from <http://www.barringtonchem.com/orig.html>
- Bermond, P. (1990) Biological effects of food antioxidants. In B. J. F. Hudson, *Food Antioxidants*. (Ch. 6, pp. 193-251) London and New York: Elsevier Applied Science.
- Beumer, R. R., te Giffel, M. C., de Boer, E., and F. M. Rombouts. (1996) Growth of *Listeria monocytogenes* on sliced cooked meat products. *Food Microbiology*. 13: 333-340.

- Chung, J. G. (1999) Effects of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on the acetylation of 2-aminofluorene and DNA-2-aminofluorene adducts in the rat. *Toxicological Science*. **51**: 202-210.
- Dharmavaram, S. R. K., Shahbazi, G., Seo, C. W., and S. A. Ibrahim. (2003) Origanox as a natural ingredient to inhibit the growth of foodborne pathogens. *Journal of Food Protection*, Poster Abstracts. **66** (P002): 59.
- Glass, K. A., and M. P. Doyle. (1989) Fate of *Listeria monocytogenes* in processed meat products during refrigerated storage. *Applied and Environmental Microbiology*. **55**: 1565-1569.
- Hänninen, M. L., Korkeala, H., and P. Pakkala. (1984) Effect of various gas atmospheres on the growth and survival of *Campylobacter jejuni* on beef. *Journal of Applied Bacteriology*. **57**: 89-94.
- Hathcock, J. N. (1995) Applications of antioxidants in physiologically functional foods: safety aspects. *Critical Reviews in Food Science and Nutrition*. **35**: 161-166.
- Iverson, F. (1999) *In vivo* studies on butylated hydroxyanisole. *Food and Chemical Toxicology*. **37**: 993-997.
- Lin, J. K., and Y.C. Liang. (2000) Cancer chemoprevention by tea polyphenols. *Proceedings of the National Science Counsel of the Republic of China B*. **24**: 1-13.
- McCall, M.R., and B. Frei. (1999) Can antioxidant vitamins materially reduce oxidative damage in humans? *Free Radical Biology and Medicine*. **26**:1034-1053.
- National Cancer Institute, Carcinogenesis Technical Report Series (1979) *Bioassay of butylated hydroxytoluene for possible carcinogenicity* [Electronic version] Retrieved May 20th, 2004, from http://ntpserver.niehs.nih.gov/htdocs/LT_rpts/tr150.pdf
- Nenadis, N., Zafiropoulou, I., and M. Tsimidou. (2003) Commonly used food antioxidants: a comparative study in dispersed systems. *Food Chemistry*. **82** (3): 403-407

- Park, S. F. (2002) The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. *International Journal of Food Microbiology*. **74**: 177-188.
- Pokorny, J. (1999) Antioxidants in Food Preservation. In: M. S. Rahman (Ed.), *Handbook of Food Preservation*, (ch.10, pp.309-339) New York: Marcel Dekker.
- Pryor, W. A., Stahl, W., and C. L. Rock. (2000) Beta carotene: from biochemistry to clinical trials. *Nutrition Review*. **58**: 39-53.
- Schuler, P. (1990) Natural antioxidants exploited commercially. In B. J. F. Hudson, *Food Antioxidants*. (Ch. 6, pp. 193-251) London and New York: Elsevier Applied Science.
- Shelef, L. A., and P. Liang. (1982) Antibacterial effects of butylated hydroxyanisole (BHA) against *Bacillus* species. *Journal of Food Science*. **47**: 796-799
- Stead, D., and S. F. Park. (2000) Roles of Fe superoxide dismutase and catalase in resistance of *Campylobacter coli* to freeze-thaw stress. *Applied and Environmental Microbiology*. **66**: 3110-3112.
- Talibart, R., Denis, M., Castillo, A., Cappellet, J. M., and G. Ermel. (2000) Survival and recovery of viable but noncultivable forms of *Campylobacter* in aqueous microcosm. *International Journal of Food Microbiology*. **55**: 263-267.
- Tenover, F.C., Knapp, J.S., Patton, C., and J.J. Plorde. (1985) Use of auxotyping for epidemiological studies of *Campylobacter jejuni* and *Campylobacter coli* infections. *Infection and Immunity*. **48**: 384-388.
- Waldenstedt, L., Inborr, J., Hansson, I., and K. Elwinger. (2003) Effects of astaxanthin-rich algal meal (*Haematococcus pluvalis*) on growth performance, caecal campylobacter and clostridial counts and tissue astaxanthin concentration of broiler chickens. *Animal Feed Science and Technology*. **108**: 119-132.
- Walis, I., Cooke, P. H., Benedict, R. C., and R. L. Buchanan. (1993) Factors affecting attachment of *Salmonella typhimurium* to sausage casings. *Food Microbiology*. **10**: 387-393.

- Williams, G. M., Iatropoulos, M. J., and J. Whysner. (1999) Safety assessment of butylated hydroxyanisole and butylated hydroxytoluene as antioxidant food additives. *Food and Chemical Toxicology*. **37** (9-1): 1027-1038.
- Yen, G., Chang, Y., Sheu, F., and H. Chiang. (2001) Isolation and characterization of antioxidant compounds from *Aspergillus candidus* broth filtrate. *Journal of Agricultural and Food Chemistry*. **49**: 1426-1431.

8. PERSONNEL CONTRIBUTING TO THIS PROJECT:

Directly funded:

M. Dworaczek, Research Technician, 100% of time and BSA students, Misses Lindsay Mooney and Jolanta Pisz part time between summer and fall of 2004.

Other personnel contributing:

P. J. Shand, P. Rogers, R. Sefton, and R. B. Pegg

9. STATEMENT OF EXPENDITURES:

A statement of expenditures prepared by Research Accounts, University of Saskatchewan has been attached.

Recommendations to Meat Industry

Based on the findings of this research and considering its context the following recommendations can be made to the meat industry.

1. There are interactions between iron, temperature and glycine betaine, which influence the survival, growth and proliferation of *Listeria monocytogenes* in processed meats. Attention must be paid to careful selection of physical conditions and chemical additives so that synergistic effects maximize the lowering of the pathogens.
2. Survival of the pathogenic bacteria on meats is influenced by osmolyte content. Glycine betaine can substantially lower *L. monocytogenes* load in processed meats and more so in temperatures higher than 4C. Glycine betaine and other osmolytes addition should be considered.
3. Temperatures, under which meats are stored, affect particular isolates of the pathogenic bacteria as to whether the bacteria will exist in free floating or attached states on the meat surface. Maintenance of temperature is crucial.
4. Presence of chelators, which can sequester divalent cations, does not help with the reduction of pathogen numbers or their decline at 4C. Addition of chelators to processed meats is unnecessary.
5. Synthetic antioxidants at ppm level have greater role in reduction of pathogen numbers. Natural antioxidants require input at parts per thousands level. Use of antioxidants is recommended.

Figure 1. Flow chart of experimental process and data collection.









